Identifying Novel Fibronectin Binding Sequences Selective to Strained and Unstrained Mechanical States

A Thesis

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Academic Faculty

By

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Abstract

Fibronectin (Fn) is an integral protein in the Extracellular matrix (ECM) and is known for its ability to lengthen under mechanical strain. It is hypothesized that this ability is based on unfolding of the protein's tertiary structure, exposing integrin-binding domains that serve as cellular binding partners. It is through this interaction that cellular processes, such as mechanotransduction, may occur. The current methods of tracking the mechanical state of Fn is through fluorescence resonance energy transfer (FRET), which is limited to *in vitro* testing and is not applicable to studying Fn in tissue. It is the goal of this study to evaluate an alternative approach to tracking the mechanical unfolding of Fn. By implementing phage display, Fn fibers in relaxed and strained mechanical states were exposed to the fuse5 library to locate high affinity binders. The results produced a peptide sequence complementary to binding sites specific to the relaxed and strained states. By applying fluorophores to these high affinity binders, molecular probes can be created to quantify mechanical strain undergone by Fn in tissue.

Introduction

As increased emphasis is placed on understanding cellular interactions and their relationships to macroscopic physiological events, the process of mechanotransduction is receiving greater attention. Mechanotransduction is the process of which cells translate mechanical stimuli from the extracellular matrix (ECM) into biochemical activity. It has been hypothesized that protein domains are a possible means of this process (1). When proteins experience linear forces, unfolding of the tertiary structure is thought to occur and cryptic domains are exposed that are otherwise hidden in the unstrained state (2). These integrin-binding domains might act as cellular binding partners that serve to initiate the biochemical responses of mechanotransduction.

Fibronectin (Fn) is an integral protein in the ECM, and a good model protein due to the tendency of its fibers to exhibit strain under physiological forces. The unfolding of its domain has been explored in past research (3). The current standard for imaging mechanical states of Fn is fluorescence resonance energy transfer (FRET) (4). FRET is the process of labeling two distinct amino acids with two complimentary fluorophores (donor and acceptor). When a high intensity light is exposed on the donor fluorophore, it is excited and releases a secondary light with a distinct wavelength. The acceptor fluorophore is chosen to accept the wavelength of light emitted by the donor fluorophore. Thus, when the secondary light reaches the acceptor fluorophore, it is excited and releases a third unique wavelength of light. Therefore, when the acceptor and donor fluorophores are located on the same Fn molecule, the FRET signal will decrease during the application of strain, due to the donor and acceptor fluorophores moving farther apart and producing less of the third unique wavelength of light. However, these studies

have been limited to *in vitro* testing due to a lack of ability to image the mechanical state of Fn *in vivo* and the necessary synthetic modifications to Fn.

Due to these limitations, a novel approach is adopted to identify the mechanical state of Fn. Phage display will be used to identify cryptic binding sites on Fn type III domains. Phage display is the cyclical process of incubating a target of interest in a population of possible target binder phages, washing away any unbound phages, and eluting and growing of strongly bound phages. This process is repeated multiple times to increase the selectivity. After several rounds of screening, a single high affinity binder should be replicated more than others for both the strained and relaxed fiber state. The results can then be validated using a simple peptide vs phage competition experiment. Therefore, this experiment should have high reproducibility and feasibility. These results will clarify the physiological process by which Fn fibers unfold. Additionally, the ability to identify Fn unfolding in living tissue will allow for more in-depth research into the mechanical states of Fn in diseases involving ECM straining.

Materials and Methods

Materials

Dyes and fluorophores were purchased from Invitrogen. Peptides were purchased from Genscript Corporation. Fuse5 phage library was obtained from Dr. G. Smith of the University of Missouri. All other materials were purchased through VWR International and Fisher Scientific.

Fibronectin Fiber Placement

Fibronectin was obtained from human plasma and diluted to a 1 mg/ml concentration. Using a pipette tip, fibers were "pulled" from the solution and deposited on to Polydimethylsiloxane (PDMS) substrate pre-affixed to a custom-machined straining device. In order to prevent fiber fracture during straining, the PDMS substrate was micro-fabricated with ridges to promote fiber suspension. The ridges were 10um by 100 um and spaced 50 um apart.

Preparation of Fibronectin Fibers

After "pulling", fibers were allowed to air dry. Any dried Fn not compromising a fiber was blocked by gelatin solution at 2 mg/ml. Upon drying of the gelatin, the fiber and surrounding environment was blocked with BSA at 10 mg/ml for 1 hour. After the BSA incubation, the PDMS substrate was strained or relaxed to the desired percentage of original strain. "Relaxed" fibers were at 93% of original strain and "strained" fibers were at 264% of original strain.

Phage Display

Prior to use of Fn fibers, $1x10^{11}$ phage from the fuse5 library were incubated for one hour on blocked PDMS. The unbound phage was grown in 10 ml of starved Tg1 E. coli cells in LB, 15 ug/ml tetracycline, and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Phage clones were retrieved through centrifugation of the cells and quantified using UV/Vis spectrometry. This phage population was used as the library for Fn fiber phage display. Fibers were exposed to $1x10^{11}$ phage for one hour, washed in PBS supplemented with 0.05% tween for 10 minutes, and eluted with 0.2M glycine at pH 2.1 before being neutralized to pH 9. The eluted cells were grown in Tg1 E. coli cells as before. In total, three series of exposures were done concurrently on relaxed and strained fibers.

Fibronectin Fiber Imaging

Images of fibers were taken by a confocal microscope based on fluorescent labeling. Fn fibers were mounted with Prolong gold solution by Invitrogen. The base Fn was created by combining unlabeled Fn and Fn labeled with AlexaFluor 488 at a 95% : 5% ratio. Additionally, this Fn was exposed to AlexaFluor 546-maleimide. This compound reacts with buried cysteine amino acids within Fn type III domains. By taking absorbance readings at both 546 and 488 wavelengths, the relative intensities of exposed cysteine amino acids and overall amount of Fn could be identified respectively. These values were normalized by calculating the quotient of the AF546 channel by the AF488 channel, to produce a single value for the degree of Fn unfolding.

Results

In order to replicate the physiological states of Fn fibers in an *in vitro* testing environment, a personalized version of Ulmer's method was followed to deposit Fn fibers upon PDMS substrate (5). Initial trials resulted in fiber fracture. A novel approach was then created to adhere the fibers to the substrate. Micro-ridges were fabricated in the substrate to promote fiber suspension and decreased fiber breakage during straining, as illustrated in Figure 1. The device was capable of delivering 300% strain, however, to promote consistency in straining, 264% strain was used in all final trials.

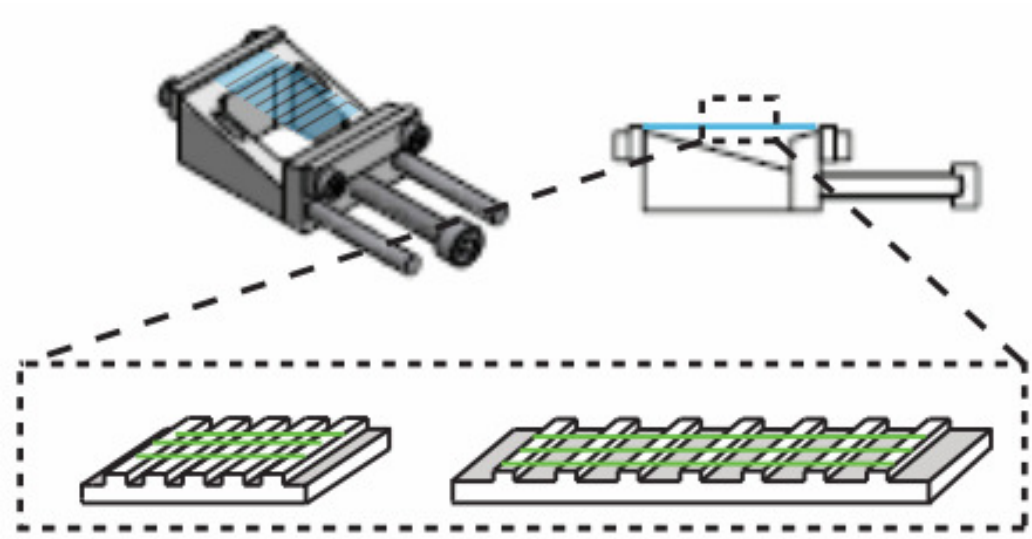

Figure 1: Process of applying controlled strain to Fibronectin fibers. Illustration of fiber deposition onto micro-fabricated PDMS substrate attached to a straining device capable of delivering 300% strain. Left, a diagram of the unstrained fiber positioning across the micro-fabricated pillars. Right, a diagram of the strained fiber positioning across the micro-fabricated pillars.

The straining of Fn fibers provided visual evidence of fiber lengthening. In order to ensure that this was due to structural unfolding of type III domains and not smaller fibers of Fn

sliding in parallel, the exposure of a "hidden" cysteine amino acid was tracked in type III domains over 25 populations. Reaction between AlexaFluor 546-maleimide labeling agent and cysteine resulted in a quantification of domain separation when imaged under AF546 and AF488 channels. Normalized intensity readings illustrated increased intensity with increasing strain and lengthening, as seen in figure 2. Upon plotting the results of the imaging, the normalized intensity was proportional to the percent strain as modeled by the linear trend line with R^2=0.6312.

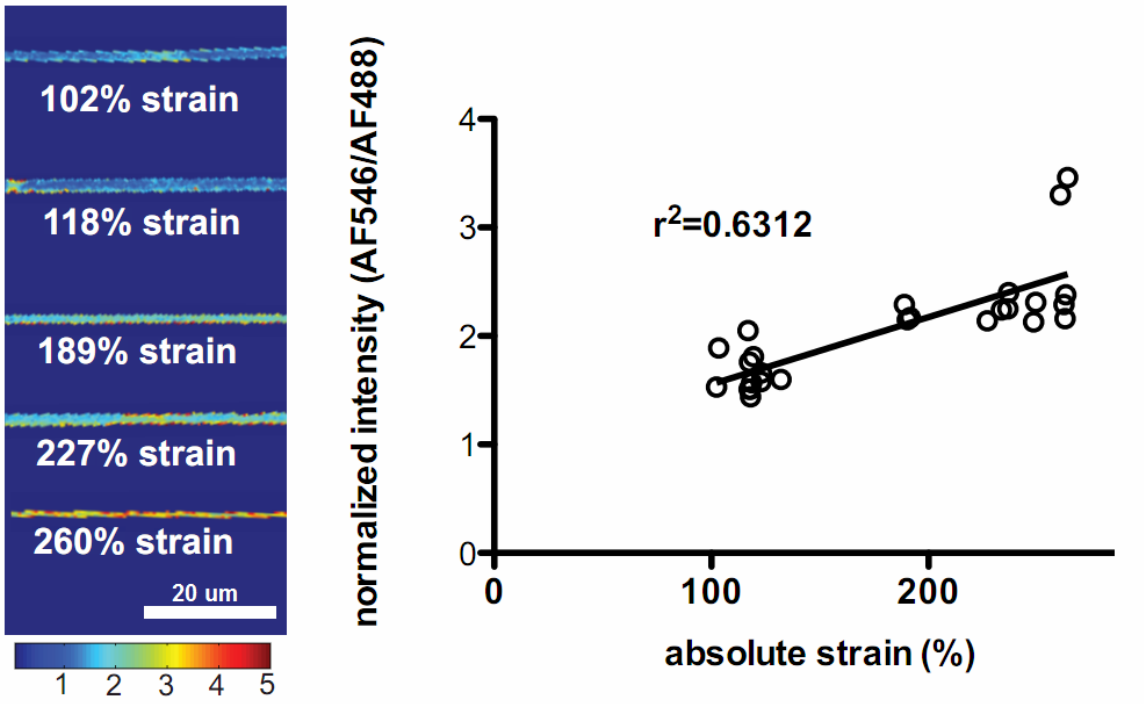

Figure 2: Justification of increased strain producing type III domain unfolding in Fibronectin. Over a sample of 25 data points, Fn fibers were exposed to AlexaFluor 546-maleimide. This compound reacted with buried cysteine amino acids within Fn type III domains. When imaged at 63X and under AF546 and AF488 channels, the quotient of the channel intensities is proportional to the amount of cysteine detected. The normalized intensity was proportional to the percent strain as modeled by the linear trend line with R^2=0.6312.

Upon justification of Fn domain unfolding, phage display was implemented to characterize new binding sites specific to the strained and unstrained fibers. Following the technique of Chen (6), a four-part system of incubation, washing, elution, and growth of the phage library was performed, as seen in figure 3, using the fuse5 library pre-screened against environmental binders of the PDMS and gelatin. The incubation step allowed any high affinity phages to bind to the Fn fiber. The washing step removed any unbound, or loosely bound, phages. The elution step removed the high affinity phages. The growing step allowed for the creation of a new library based on the high affinity binders collected from the elution.

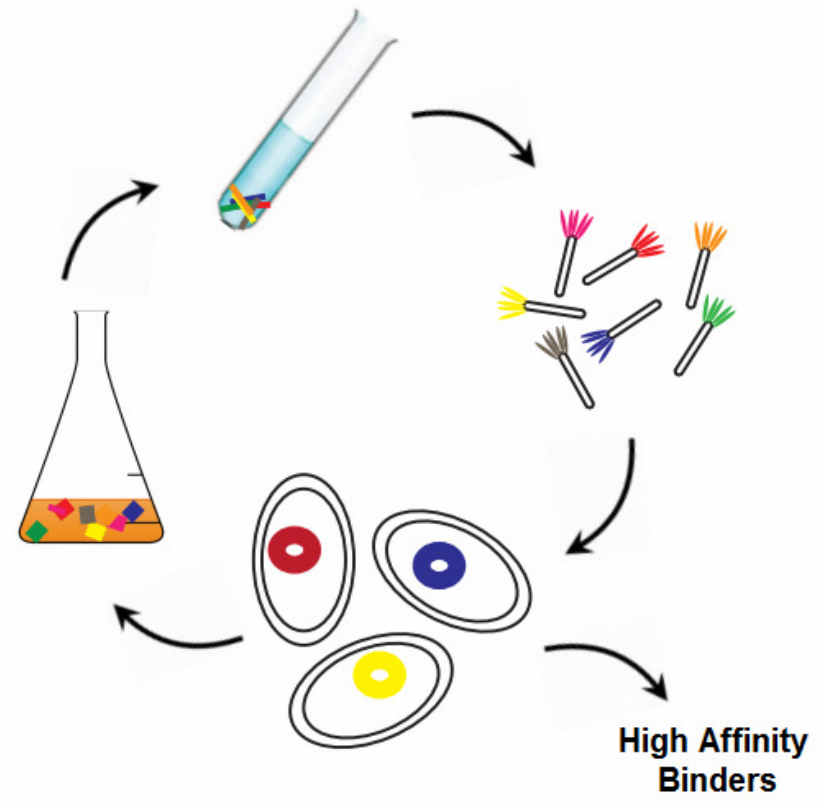

Figure 3: Process of screening for cellular binding site partners of strained and unstrained Fibronectin fibers. In the identification process of phage display, the Fn fibers are exposed to the fuse5 phage library. Upon removal of the supernatant and washing, bound phages are eluted and grown. After multiple cycles, only high affinity binder phages remain.

After finishing the initial phage display process, eight interesting clones were found. Fibers were then exposed to each of these clones at least three times via phage display. Each exposure was ran with a relaxed fiber at 93% of the original strain and a strained fiber at 264% of the original strain. Upon completion of these exposures, tittering was used to quantify the results. Of the eight clones investigated, five proved to have strain-specific binding with tittering results of over 10^7 CFU, as seen in figure 4. Two particular clones were selected of high interest. Clone 2 had the highest binding affinity to strained fibers at $3.1x10^7$ CFU as compared with relaxed fibers at $1.4x10^6$ CFU. Clone 3 had the second highest binding affinity for relaxed fibers, but had a much smaller standard deviation than the highest relaxed binder: clone four. It bound selectively to relaxed fibers at $1.4x10^7$ as compared to strained fibers at $1.8x10^6$ CFU.

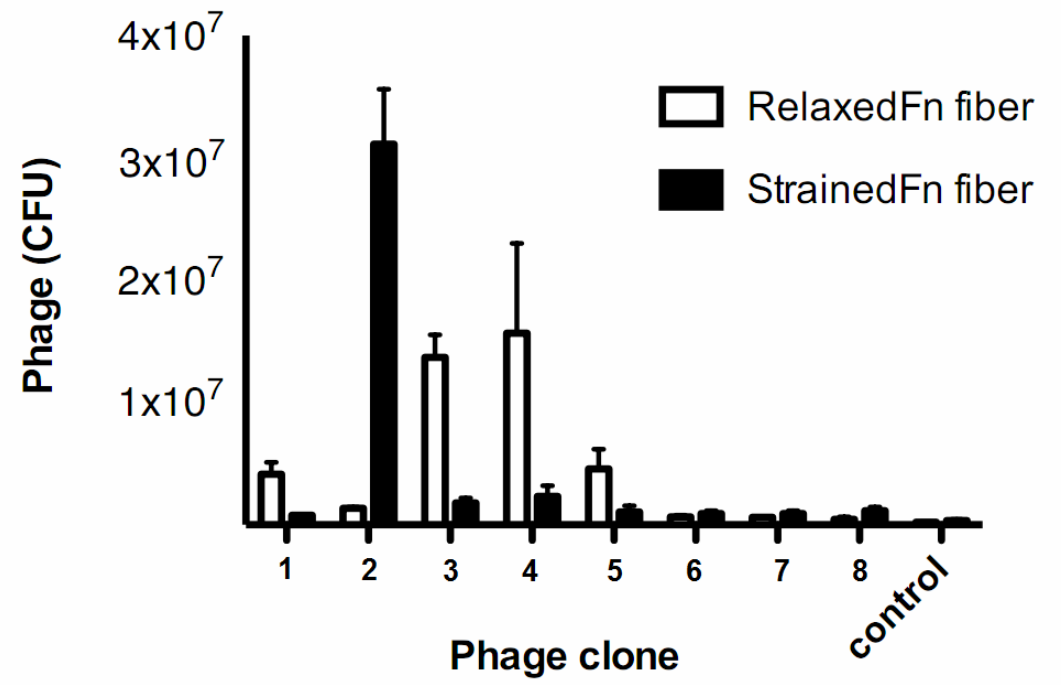

Figure 4: Results of exposing relaxed and strained Fibronectin fibers to eight different phage clones at $1x10^{11}$ CFU. The relaxed Fn fibers, held at 93% of original strain, bound most selectively by clone 3 and clone 4. The strained Fn fibers, exposed to 264% of original strain, bound most selectively to clone 2. The control phage population was the resulting library of phages after three phage screenings. Three or more samples were ran for each clone.

After discovering the affinity of clone 3 to relaxed fibers, a binding competition was ran to asses its selectivity. Relaxed fibers were co-incubated in phages of clone 3 and varying levels of clone 3's phage-free peptide equivalent. Ideally, an increase in the levels of soluble peptide equivalents to the clone 3's binding site will increase the competition for the limited sites available per fiber and decrease the number of clone 3 phages that successfully bind. Based on a one-way ANOVA with a P-value below an α-value of 0.05, statistically significant differences were recognized between the competition experiment with 0 uM of phage-free peptides and 10 uM of phage-free peptides, as well as 0 uM of phage-free peptides and 100 uM of phage-free peptides, as seen in figure 5. This illustrates a high degree of specificity of clone 3 for relaxed Fn fibers.

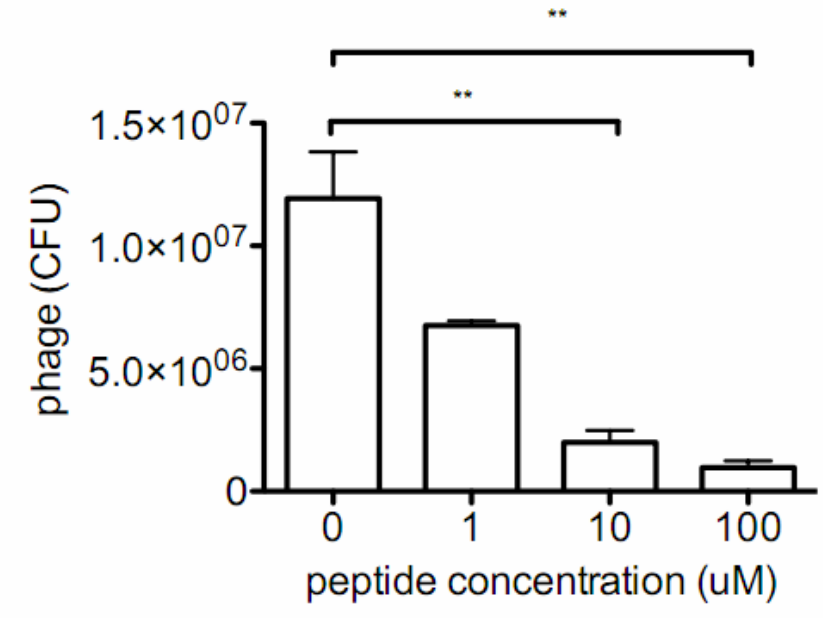

Figure 5: Binding competition between selective phage clone 3 and 0, 1, 10, and 100 uM phage-free peptides exposed to relaxed (93% original strain) Fibronectin fibers. Phage clone 3 was incubated against its phage-free form. Statically significant differences can be seen between the competitive incubation of clone 3 relative to 0 uM and 10 uM as well as to 0 uM and 100 uM of its phage-free form. This statistical decrease illustrates competitive inhibition is occurring between phage clone 3 and its phage-free form. More than three samples were ran. Error bars are based on standard error of the mean. Statistical analysis was performed with a one-way ANOVA with a P-value below an α-value of 0.05.

In addition to the competition experiment ran for clone 3, one was also ran for clone 2. Strained fibers were co-incubated in phages of clone 2 and varying levels of clone 2's phage-free peptide equivalent. Ideally, an increase in the levels of soluble peptides equivalent to the clone 2's binding site will increase the competition for the limited sites available per fiber and decrease the number of clone 2 phages that successfully bind. Based on a one-way ANOVA with a P-value below an α-value of 0.05, statistically significant differences were recognized between the competition experiment with 0 uM of phage-free peptides and 1 uM of phage-free peptides, 0 uM of phage-free peptides and 10 uM of phage-free peptides, as well as 0 uM of phage-free peptides and 100 uM of phage-free peptides, as seen in figure 6. This illustrates a high degree of specificity of clone two 2 strained Fn fibers.

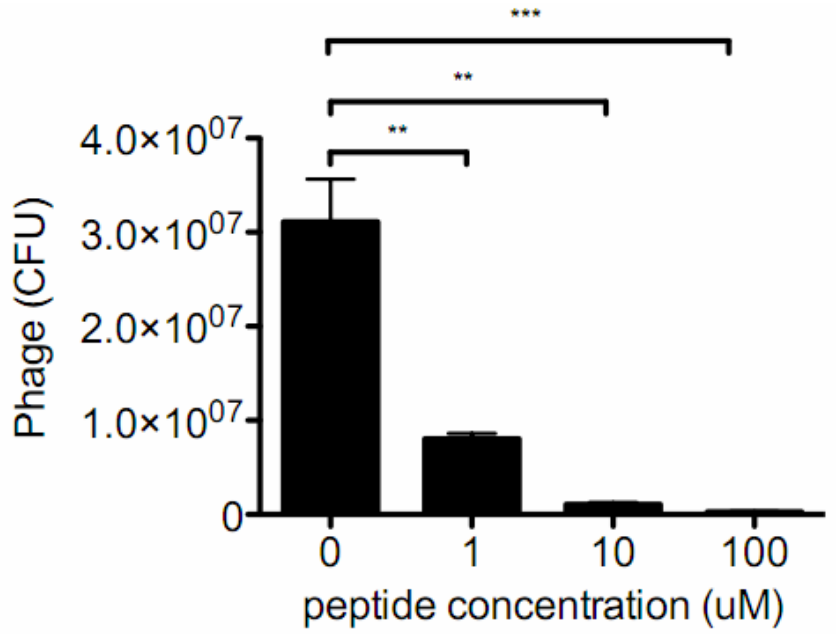

Figure 6: Binding competition between selective phage clone 2 and 0, 1, 10, and 100 uM phage-free peptides exposed to strained (264% original strain) Fibronectin fibers. Phage clone 2 was incubated against its phage-free form. Statically significant differences can be seen between the competitive incubation of clone 2 relative to 0 uM and 10 uM of its phage-free form, to 0 uM and 10 uM of its phage-free form, and to 0 uM and 100 uM of its phage-free form. This statistical decrease illustrates competitive inhibition is occurring between phage clone 3 and its phage-free form. More than three samples were ran. Error bars are based on standard error of the mean. Statistical analysis was performed with a one-way ANOVA with a P-value below an α-value of 0.05.

To further measure the selectivity of clone 3 to relaxed fibers, binding competition was ran with respect to scrambled versions of the clone three sequence. Ideally, an increase in the level of soluble scrambled peptides will have no affect on the number of bound phages, due to dissimilar binding sites. This relationship is confirmed by the data, based on a one-way ANOVA with a P-value below an α-value of 0.05. Statistically significant differences were recognized between the competition experiment with 0 uM of phage-free peptides and 100 uM of phage-free peptides, as well as 0 uM of phage-free peptides and 100 uM of phage-free peptides, as seen in figure 7. However, no statistically significant differences were recognized between the competition experiment with 0 uM of phage-free peptides and 100 uM of phage-free scrambled peptides.

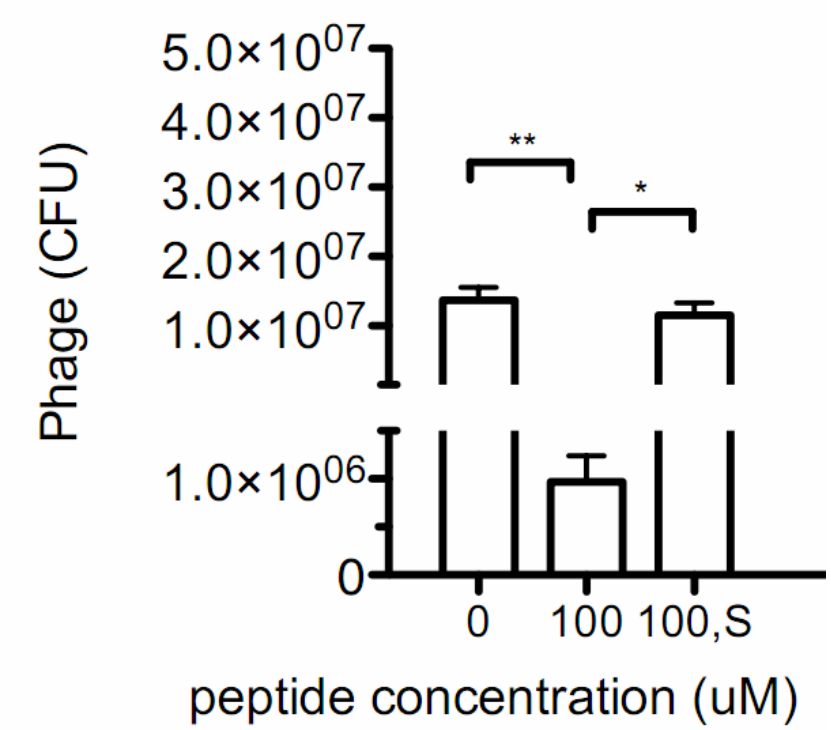

Figure 7: Binding competition between selective phage clone 3, 100 uM phage-free peptides, and 100 uM scrambled phage-free peptides exposed to relaxed (93% original strain) Fibronectin fibers. Peptide scrambling is the process of rearranging the sequence of peptides. Phage clone 3 was incubated against its phage-free form and its scrambled phage free form. Statically significant differences can be seen between the competitive incubation of clone 3 relative to 0 uM and 100 uM of its phage-free form and relative to 100 uM of its scrambled phage-free form and 100 uM of its phage-free form. This statistical analysis illustrates competitive inhibition is occurring between phage clone 3 and its phage-free form, but not between phage clone 3 and its scrambled phage free form . More than three samples were ran. Error bars are based on standard error of the mean. Statistical analysis was performed with a one-way ANOVA with a P-value below an α-value of 0.05.

In addition to running a competition experiment with scrambled peptides of clone 3, the same experiment was ran with clone 2 phages. Ideally, an increase in the level of soluble scrambled peptides will have no affect on the number of bound phages, due to dissimilar binding sites. This relationship is confirmed by the data, based on a one-way ANOVA with a P-value below an α-value of 0.05. Statistically significant differences were recognized between the competition experiment with 0 uM of phage-free peptides and 100 uM of phage-free peptides

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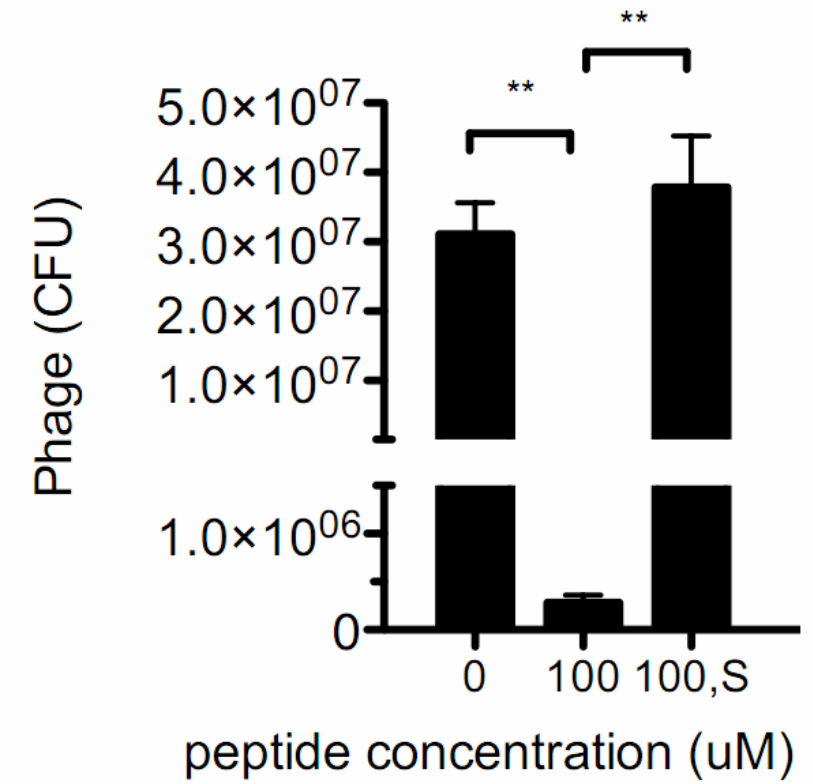

Figure 8: Binding competition between selective phage clone 2, 100 uM phage-free peptides, and 100 uM scrambled phage-free peptides exposed to strained (264% original strain) Fibronectin fibers. Phage clone 2 was incubated against its phage-free form and its scrambled phage free form. Statically significant differences can be seen between the competitive incubation of clone 2 relative to 0 uM and 100 uM of its phage-free form and relative to 100 uM of its scrambled phage-free form and 100 uM of its phage-free form. This statistical analysis illustrates competitive inhibition is occurring between phage clone 2 and its phage-free form, but not between phage clone 2 and its scrambled phage free form . More than three samples were ran. Error bars are based on standard error of the mean. Statistical analysis was performed with a one-way ANOVA with a P-value below an α-value of 0.05.

Discussion

The major goal of this experiment was to provide the means to establish a novel approach to analyzing Fn unfolding through finding specific high affinity binders for folded and unfolded fibers. Whereas past approaches used FRET, and were only applicable for *in vitro* experiments, labeling these high affinity binders with fluorescent probes would allow future research to study Fn in *in vivo* and *ex vivo* tissue samples.

To achieve this approach, controlled methods of unfolding fibers were used in conjunction with phage display to pan for high affinity binding peptides. Upon multiple exposures to the fuse5 library, significant results were found. As seen in figure 4, phage clone 3 showed a highly selective affinity to binding relaxed (folded) fibers and phage clone 2 showed a highly selective affinity to binding strained (unfolded) fibers. This correlation was further strengthened by testing clones against soluble versions of their peptide sequence in competition. In these experiments, increased levels of soluble peptides statistically decreased the number of phages bound due to competition. However, when tested against scrambled versions of the peptide sequence, there was no affect on binding. This supports the selectivity of clones 3 and 2 for relaxed and strained fibers, respectively. These results supported the goal of identifying selective binding sites specific to folded and unfolded mechanical states.

The identification of these sequences will aid in further research in the field of ECM study involving Fn. By attaching different fluorescent probes to the strained and unstrained binding peptide sequences, researchers will be able to know the conformational state of target Fn. Additionally, through further experimentation with probes, a calibration curve could be produced that could relate the normalized intensities between the two different fluorescent

probes and the known fiber strain in a controlled environment. This could then be used outside of controlled environments to measure the percent strain on the experimental Fn.

Conclusion

The purpose of this experiment was to identify binding sites on Fn fiber type III domains, specific to relaxed and strained fibers. Fn fibers were deposited on substrate capable of inducing up to 300% strain. By running phage display on relaxed and strained fibers with respect to the fuse5 library, high affinity binders for relaxed and strained states were identified. Upon completion of competition experiments against soluble phage-free versions of the clone's peptides and scrambled versions of the clone's peptide sequence, their specificity was confirmed. By labeling these relaxed and strain specific binders with two different fluorophores, image analysis of fibers will allow for quantification of the degree of strain present.

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